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(57) Abstract

A method of selecting anti-aggregation molecules with chaperone-like activity that have characteristics including binding to a native target molecule epitope with a high binding constant and which are non-inhibitory to the biological activity of the target molecule when bound. The method includes the steps of mixing a denatured target molecule with a presumptive anti-aggregation molecule and then determining if the target molecules are prevented from self- or induced-aggregation. The nonaggregated target molecule coupled to the anti-aggregation molecule is then tested for bioactivity.

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PREVENTION OF PROTEIN AGGREGATION

TECHNICAL FIELD

The present invention relates to the use of monoclonal antibodies, genetically engineered antibody fragments and small peptides which mimic antigen binding sites on the antibody for the prevention of protein aggregation *in vivo* and *in vitro*.

BACKGROUND OF THE INVENTION

When proteins are synthesized they generally must fold and assemble into a three dimensional form to be active. Initially, it was thought that proper folding was inherent in the amino acid sequence. Recent work has shown that additional proteins, now referred to as molecular chaperones, are required to mediate the folding process or unregulated aggregation of the polypeptides will occur preventing the formation of functional proteins (Goloubinoff et al., 1989; Welch, 1993). However, despite the existence of chaperones, aggregation of protein still occurs *in vivo* and can contribute to, or cause, various disease states.

Other factors must contribute to the occurrence of aggregation. These factors can include mutations of naturally occurring chaperones inhibiting function or allowing function with low efficiency (Wetzel, 1994). Further, "pathological" chaperones have been found which have been defined as "a group of unrelated proteins that induce beta-pleated conformation in amyloidogenic polypeptides" (Wisniewski and Frangione, 1992). It would be useful to be able to replace or augment the activity of the chaperones where necessary and to counteract the

activity of pathological chaperones when present. Additionally, prion diseases have been shown to be caused by, and/or associated with, a conformational protein change wherein a protein normally having an alpha helix conformation is converted to beta strands which are collected into beta sheets (Prusiner, 1995).

Protein aggregation is of major importance in biotechnology for the *in vitro* production of recombinant proteins. *In vitro* aggregation limits the protein stability, solubility and yields in production of recombinant proteins. In cells during production of recombinant proteins, aggregation is a major impediment of recombinant proteins leading to formation of inclusion bodies in the host cells (DeYoung et al, 1993; Wetzel, 1994; Vandenbroeck et al., 1993).

Further, *in vivo* protein aggregation or precipitation is the cause, or an associated pathological symptom, in amyloid diseases such as Down's syndrome, Alzheimer's disease, diabetes and/or cataracts, and in other disorders (DeYoung et al., 1993; Haass and Selkoe, 1993; Wetzel, 1994; Prusiner, 1995).

Several peptides including β-amyloid, have been shown to spontaneously self-associate, or aggregate, into linear, unbranched fibrils in serum or in isotonic saline (Banks and Kastin, 1992; Haass and Selkoe, 1993). At least fifteen different polypeptides are known to be capable of causing *in vivo* different forms of amyloidosis via their deposition in particular organs or tissues as insoluble protein fibrils. Iron, zinc, chromium or aluminum can participate in this aggregation (Bush et al., 1994).

Molecular chaperones were initially recognized as stress proteins produced in cells

requiring repair. In particular, studies of heat shock on enzymes led the way to the discovery of molecular chaperones that function not only during cellular stress but normally to produce properly folded proteins. The heat shock model is still one of the models of choice in studying molecular chaperones (Welch, 1993; Goloubinoff et al., 1989).

Molecular chaperones are a ubiquitous family of proteins that mediate the post-translational folding and assembly of other unrelated proteins into oligomeric structures. They are further defined as molecules whose functions are to prevent the formation of incorrect structures and to disrupt any that form. The chaperones non-covalently bind to the interactive surface of the protein. This binding is reversed under circumstances that favor the formation of the correct structure by folding. Chaperones have not been shown to be specific for only one protein but rather act on families of proteins which have the same stoichiometric requirements, i.e specific domains are recognized by chaperones. This does not provide the specificity required for therapeutic activity.

Further uses and descriptions of molecular chaperones are set forth in PCT published international patent applications 93/11248, 93/13200, 94/08012 and 94/11513 incorporated herein by reference and in particular 94/08012 page 2 line 20 through page 5, line 14.

PCT published international patent application 93/11248 discloses the use of a chaperone in cell culture to promote efficient production of protein in transformed cells by co-expression of the chaperone molecule. This disclosure does not provide specificity as to which proteins are protected except through co-expression with the wanted protein nor does

it provide information on how to use chaperones therapeutically.

PCT published international patent application 93/13200 discloses the use of a chaperone in a purification step for a recombinant protein isolated from a cell culture and also a fusion protein of the chaperone and recombinant protein. This disclosure also does not provide specificity as to which proteins are protected except through co-expression with the wanted protein nor does it provide information on how to use chaperones therapeutically.

PCT published international patent application 94/08012 discloses the use of a chaperone in cell culture to promote increased secretion of an overexpressed gene product in a host cell. This disclosure does not provide specificity as to which proteins are protected except through co-expression with the wanted protein nor does it provide information on how to use chaperones therapeutically.

PCT published international patent application 94/11513 discloses the use of a vector containing a molecular chaperone for treating neoplasms. This disclosure does not provide specificity as to which proteins are protected except through co-expression with the wanted protein nor does it provide information on how to use chaperones therapeutically to treat diseases or syndromes which involve protein aggregation.

In each of the aforementioned publications, the chaperones did not bind to native proteins and did not redissolve aggregated proteins.

Recent reports suggest that monoclonal antibodies (mAb) can have chaperone-like activity. The feasibility of using monoclonal antibodies to assist in the *in vitro* refolding process of guanidine-denatured S-protein was reported recently (Carlson and

Yarmush, 1992). Previously, Blond and Goldberg (1987) used mAbs as a tool in the identification and characterization of folding steps that involve the appearance of local native-like structures in B_2 subunit of tryptophan-synthase. Since the mAb is epitope specific, the use of a mAb provides more specificity than molecular chaperones. Monoclonal antibodies can be sought and engineered (Haber, 1992) that bind to the particular epitope in the protein of interest that is involved in the folding process.

The main difference between mAbs and molecular chaperones is that the latter does not bind to native proteins and is capable of interacting with many different polypeptide chains without exhibiting an apparent sequence preference (Goloubinof et al., 1989). Moreover, chaperones suppress aggregation but do not redissolve aggregate proteins already present. Similar behavior was recently reported for α-crystalin which, similar to other chaperones, does not react with active proteins, but forms a stable complex with denaturing or partially unfolded proteins, stabilizing against further aggregation (Rao et al., 1994).

Aggregated amyloid β-protein (βA4) is a major constituent of the abnormal extracellular amyloid plaque that characterizes the brains of victims of Alzheimer's disease (AD) (Haass and Selkoe, 1993). *In vitro* studies have shown that some of the metal ions found in biological systems, i.e. Fe, Al and Zn, can accelerate the aggregation process dramatically. The presence of "pathological" chaperones (Wisniewski and Frangione, 1992) and the above listed metals (Mantyh et al., 1993; Fraser et al., 1993) as proposed risk factors in Alzheimer's disease, favor β-amyloid cascade aggregation. If the interaction between the metal ion and the β-amyloid can be interrupted or prevented, then metal-induced

aggregation can be reduced or eliminated. However, just binding a mAb at this site might prevent the metal-induced aggregation but would not allow normal functioning of the protein.

It would therefore be useful to develop the appropriate mAb with chaperone-like characteristics directed to the appropriate epitope on the β-amyloid molecule in order to prevent the accelerated metal-induced aggregation.

In prion diseases it has been shown that the pathologic "scrapie prion protein" propagates itself by contacting normal prion protein and causing them to unfold and flip from their usual conformation to the scrapie prion protein state, a beta-pleated sheet. This initiates a cascade effect. It would therefore be useful to be able to interrupt the "scrapie-prion" cascade.

Further, it would be particularly useful to be able to develop a mAb as needed that prevents the aggregation of proteins/enzymes *in vivo* but that still allows the proteins/enzymes to function even when bound to the mAb.

With the advent of recombinant DNA methods for the production of proteins in bacteria methods are needed to prevent aggregation during production and harvesting. Chaperones have been shown to suppress aggregation during the folding of a number of protein *in vitro* and co-expression of chaperones can also suppress formation of inclusion bodies by some foreign proteins *in vivo* as described herein. However, despite the use of chaperones, protein aggregation can still occur during production and harvesting.

Still further, it is not always possible to isolate the appropriate chaperone for preventing aggregation of a molecule and to utilize it as a therapeutic. The availability of engineering and

selecting mAbs and delivery systems for mAb makes it useful to develop specific mAb to serve as therapeutic chaperones.

SUMMARY OF THE INVENTION AND ADVANTAGES

According to the present invention, a method is provided of selecting an anti-aggregation molecule such as a monoclonal antibody, a genetically engineered antibody fragment or a peptide which mimics the binding site of an antibody. These anti-aggregation molecules are able to bind to a native target molecule epitope with a high binding constant and are selected to be non-inhibitory to biological activity of the target molecule.

The present invention further provides a method of treating a protein aggregation disease by creating an expression vector comprising nucleic acid including a sequence which encodes in expressible form the human form of the anti-aggregation molecule that binds to a native target molecule, an aggregating protein, and which prevents aggregation and allows biological activity of the target molecule.

The present invention also provides an expression vector comprising nucleic acid including a sequence which encodes in expressible form the human form of the anti-aggregation molecule that binds to a native target molecule, an aggregating protein, and which prevents aggregation and allows biological activity of the target molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIGURE 1 is a bar graph of the temperature-dependence of enzymic activity of Carboxypeptidase A (CPA); the residual enzymic activity of CPA after one hour incubation at increasing temperatures was measured using esterase substrate;

FIGURE 2 is a bar graph of the time course of denaturation of Carboxypeptidase A after exposure at 50°C; the residual esterase (□) and peptidase (cross-hatch) enzymic activity of CPA was measured at two intervals of incubation at 50°C; the amount of residual soluble enzyme was determined by sandwich ELISA (■);

FIGURE 3 is a bar graph of the enzymic activity of Carboxypeptidase A retained after exposure to 50°C for one hour in the presence of monoclonal antibody CP_{10}; the immunocomplexation of CPA with increasing amounts of CP_{10} was performed before exposure at 50°C for one hour; the residual peptidase (■) and esterase (□) enzymic activity of CPA was measured;

FIGURE 4 is a bar graph of the effect of epitope location on the maintenance of the enzymic activity of heat-exposed Carboxypeptidase A; increasing amounts of monoclonal antibodies CP_{10} (cross-hatch) and CP_9 (□) and unrelated IgG (■) were added to CPA before exposure to 50°C for one hour and esterase enzymic activity was measured;

FIGURE 5 is a bar graph of the prevention of aggregation of Carboxypeptidase A by monoclonal antibody CP_{10}; aggregation of CPA, in the presence (double cross-hatch bars) and in the absence (single cross-hatch bars) of antibodies, was followed by

determination of amount of mAb bound to coated CPA in a competitive ELISA; the absorbance at 495 nm obtained in the absence of added soluble CPA was set at 100% for bound antibody; the soluble CPA, before heat exposure, competes with the coated CPA for antibody binding, leading to decrease in amount of antibody bound (60%) (diagonal lines);

FIGURE 6 is a bar graph showing thermal aggregation of Carboxypeptidase A and its suppression by monoclonal antibodies CP_{10} and CP_9; aggregation of Carboxypeptidase A after exposure at 50°C for one hour in the absence (□) of monoclonal antibodies and in the presence of CP_{10} (cross-hatch) and CP_9 (■) was followed by determination of amount of antibody bound by sandwich ELISA; maximum binding (100%) was considered the amount of antibody bound to CPA before exposure to aggregation conditions;

FIGURE 7 is a pair of graphs (**A** and **B**) showing aggregation of β-amyloid (1-40) in the absence (cross-hatch) and in the presence (□) of monoclonal antibodies AMY-33 (**A**) and 6F/3D (**B**) followed by ELISA; (1) β-amyloid alone, (2) β-amyloid + 50 MM heparan sulfate, (3) β-amyloid + 10^{-3}M $AlCl_3$; (4) β-amyloid + 10^{-3} M $ZnCl_2$;

FIGURE 8 is a pair of graphs (**A** and **B**) showing a Thioflavin T based fluorimetric assay of β-amyloid aggregation (■) in the presence of mAb AMY-33 (□) and an unrelated antibody (cross-hatch) with (**A**) an emission spectra of Thioflavin T bound to fibrillar β-amyloid peptide (upper curve) and in the presence of antibody AMY-33 the immunocomplex with βA4 (lower curve) and (**B**) shows an increase in Thioflavin T fluorescence bound to β-amyloid peptide (■) as a

function of incubation time of the peptide as measured after three hours, three days and seven days wherein immunocomplexation with mAb AMY-33 prevents the increase in Thioflavin T fluorescence (□), while unrelated antibody (cross-hatch) did not interfere with its fluorescence; and

FIGURE 9 is a bar graph showing aggregation in the presence of various monoclonal antibodies to β-amyloid peptide in the absence of soluble β-amyloid (100%, maximal binding) and calculated by comparison with the binding of the same monoclonal antibodies to the residual soluble β-amyloid peptide remaining after incubation in the absence of the respective antibody.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides a method of selecting monoclonal antibodies, genetically engineered antibody fragments and small peptides which mimic binding sites of the antibodies that prevent aggregation of a native target molecule, which may aggregate and is in general a protein, and yet do not inhibit bioactivity when bound to the target molecule. These anti-aggregation molecules with chaperone-like activity are able to bind to a native target molecule epitope with a high binding constant and yet are selected to be non-inhibitory to the biological activity of the target molecule when bound.

The method can include culturing an appropriate host cell transformed with DNA encoding the target molecule. The host cell chosen will express the target molecule in aggregated form. Examples of such cells are set forth in PCT published international patent application 93/11248, 93/13200 and 94/08012. Alternatively, the appropriate

recombinant target molecule can be purchased or a naturally occurring molecule can be isolated or purchased.

The expressed target molecule is recovered and denatured thereby also deaggregating the molecules if they are aggregated. The denatured target molecule is mixed with the presumptive anti-aggregation molecule such as a monoclonal antibody, genetically engineered antibody fragment or small peptide which mimics an antibody binding site generally as set forth in PCT pending application 93/13200 and under conditions which allow for renaturing and for self-aggregation such as temperature, pH or interaction with other aggregation-inducing agents. Mixtures which produce non-aggregated target molecules are selected. It is then determined if the nonaggregated target molecules are bioactive even in the presence of, and bound to, the presumptive anti-aggregation molecule.

In addition, the anti-aggregation molecule is screened for its ability to dissolve already aggregated proteins. The aggregated proteins are mixed with the anti-aggregation molecules under physiological conditions. Mixtures with non-aggregated molecules are selected. It is then determined if the nonaggregated target molecules are bioactive even in the presence of, and bound to, the presumptive anti-aggregation molecule.

The antibodies, or peptide mimicking the binding site, bind to an epitope on the target molecule which is a region responsible for folding or aggregation. In addition the anti-aggregation molecule is selected only if it does not show immune cross reactivity with other proteins with proximity to the target molecules under the same conditions employed in the bioactivity tests; that is, molecules

which are found in the cell near the target molecule or molecules with sequences similar to the target molecules.

After the identification of the anti-aggregation molecules has been completed, it is possible in one embodiment to utilize two or more to prevent or reverse aggregation. They can be used concurrently to increase their chaperone-like effect, if their respective target epitopes are not overlapping and if, in binding to the target molecule, they do not interfere with each other.

Bioactivity is tested as is appropriate for the target molecule. For example, enzymatic activity of the target molecule for its substrate can be measured. Assays which measure *in vitro* enzymatic bioactivity are well known to those skilled in the art.

In one embodiment of the method, the target molecule is β-amyloid and the monoclonal antibody is an anti-β-amyloid monoclonal. Alternatively, a genetically engineered antibody fragment as described hereinbelow can be used or a small peptide which mimics the antigen binding site on the target molecule can be used. The antigen binding site of an antibody can be determined as is known in the art as for example from x-ray crystallography, DNA sequences or the like.

The method has also been demonstrated with carboxypeptidase A as set forth in the Examples hereinbelow.

Other peptides or proteins with evidence of self aggregation can also be used in the present invention such as amylin (Young et al., 1994); bombesin, caerulein, cholecystokinin octapeptide, eledoisin, gastrin-related pentapeptide, gastrin tetrapeptide, somatostatin (reduced), substance P; and

peptide, luteinizing hormone releasing hormone, somatostatin N-Tyr (Banks and Kastin, 1992), and prion protein (PrP).

Once an appropriate monoclonal antibody with chaperone-like activity is found or engineered or a peptide with the appropriate configuration, the present invention provides for its use therapeutically to prevent or reduce protein aggregation *in vivo*. In an embodiment, the prevention of β-amyloid aggregation or prion-associated protein aggregation is undertaken.

A method of treating a protein aggregation disease intracellularly includes the steps of preparing (Haber, 1992; Harlow & Lane, 1988) or selecting an anti-aggregation molecule as set forth herein, such as a monoclonal antibody, genetically engineered monoclonal antibody fragment or peptide that mimics the binding site of an antibody, that binds to an aggregating protein which is the cause of a disease and which prevents aggregation and yet allows the protein to be bioactive even while bound. This molecule can be referred to as an anti-aggregation molecule with chaperone-like activity. An expression vector is created comprising nucleic acid including a sequence which encodes in expressible form the anti-aggregation molecule. The expression vector is then delivered to the patient.

In an embodiment a human monoclonal antibody that binds to an aggregating protein and which prevents aggregation is utilized. In a further embodiment the monoclonal antibody (mAb) is an anti-β-amyloid and is selected from the group consisting of AMY-33 which recognizes an epitope spanning amino acids 1-28 of β-amyloid, mAbs 6C6 and 10D5, which recognize an epitope spanning the amino acid residues 1-16 of β-amyloid peptide, and mAbs 2H3 and 1C2,

directed to the regions comprising peptides 1-12 and 13-28, respectively.

Work by Dueñas et al. (1994) and Marasco et al. (1993) have shown that single chain monoclonal antibodies are efficient for intracellular expression in eukaryotic cells. The single chain monoclonal antibody is composed of an immunoglobulin heavy chain leader sequence and heavy and light chain variable regions that are joined by an interchain linker. The molecule is small, approximately 28 kDa with high-affinity ligand-binding capability and minimal assembly requirement. The antibody can be directed to the relevant cellular compartment using classical intracellular-trafficking signals. Marasco et al. (1993) have shown that such antibodies are not toxic to the cells and function when expressed in the cell.

The production of expression vectors is well known to those skilled in the art. In a preferred embodiment, the expression vector is constructed using the methodology as set forth by Dueñas et al. (1994), PCT pending application 94/11513. Methods not explicitly set forth are performed as generally set forth in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1992), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989).

Such vectors are known or can be constructed by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences. Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the nucleic acids in a different form. Phagemids are a specific example of such beneficial vectors because they can be used either as plasmids or as bacteriophage vectors.

Examples of other vectors include viruses such as bacteriophages, baculoviruses and retroviruses, DNA viruses, cosmids, plasmids, liposomes and other recombination vectors. The vectors can also contain elements for use in either procaryotic or eucaryotic host systems. One of ordinary skill in the art will know which host systems are compatible with a particular vector.

The expression vector can be a virus. Further the virus can be an RNA virus such as a disabled retro virus or a retroviral shuttle vector. The expression vector can also be vaccinia virus or an adenovirus. The expression vector can also be a plasmid. In a preferred embodiment wherein β-amyloid is the targeted molecule the expression vector is selected that is known to target the central nervous system.

In the present invention, the expression vector for use as a therapeutic agent comprises a nucleic acid including at least one sequence which encodes in expressible form an anti-aggregation molecule, which molecule binds to an aggregating protein that is the cause of a disease and which prevents aggregation but does not interfere with bioactivity. In an embodiment the expression vector includes the sequence for a human monoclonal antibody that is an anti-β-amyloid monoclonal antibody with heparan-like characteristics. In a further preferred embodiment, the expression vector includes the sequence for the single chain monoclonal antibody of the above anti-β-amyloid mAb.

A specific example of a DNA viral vector for introducing and expressing recombinant sequences is the adenovirus derived vector Adenop53TK. This vector expresses a herpes virus thymidine kinase (TK) gene for either positive or negative selection and an

expression cassette for desired recombinant sequences. This vector can be used to infect cells that have an adenovirus receptor. This vector, as well as others that exhibit similar desired functions, can be used to treat a mixed population of cells and can include, for example, an *in vitro* or *ex vivo* culture of cells, a tissue or a human subject.

Additional features can be added to the vector to ensure its safety and/or enhance its therapeutic efficacy. Such features include, for example, markers that can be used to negatively select against cells infected with the recombinant virus such as antibiotic sensitivity. Negative selection is therefore a means by which infection can be controlled because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce altered forms of the viral vector or recombinant sequence, cellular transformation will not occur. Features that limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are specific for the desired cell type.

In addition, recombinant viral vectors are useful for *in vivo* expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be

produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

As described above, viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. The vector to be used in the methods of the invention will depend on desired cell type to be targeted and will be known to those skilled in the art. For example, if breast cancer is to be treated then a vector specific for such epithelial cells would be used. Likewise, if diseases or pathological conditions of the nervous system are to be treated, then a viral vector that is specific for neural cells such as neurons, oligodendroglia and the like and their precursors, preferably for the specific type of hematopoietic cell, would be used.

Retroviral vectors can be constructed to function either as infectious particles or to undergo only a single initial round of infection. In the former case, the genome of the virus is modified so that it maintains all the necessary genes, regulatory sequences and packaging signals to synthesize new viral proteins and RNA. Once these molecules are synthesized, the host cell packages the RNA into new viral particles which are capable of undergoing further rounds of infection. The vector's genome is also engineered to encode and express the desired recombinant gene. In the case of non-infectious viral vectors, the vector genome is usually mutated to

destroy the viral packaging signal that is required to encapsulate the RNA into viral particles. Without such a signal, any particles that are formed will not contain a genome and therefore cannot proceed through subsequent rounds of infection. The specific type of vector will depend upon the intended application. The actual vectors are also known and readily available within the art or can be constructed by one skilled in the art using well-known methodology as well as many being commercially available.

The expression vector containing the sequence for the anti-aggregation molecule may be administered to mammals, including humans, by any route appropriate to the condition being treated and in several ways. Suitable routes include oral, rectal, nasal, topical, vaginal and parenteral. It will be appreciated that the preferred route may vary with, for example, the condition of the recipient and the type of treatment envisaged.

If viral vectors are used, for example, the procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site. However, local administration can provide a quicker and more effective treatment, administration can also be performed by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into spinal fluid can also be used as a mode of administration, especially in the case of neuro-degenerative diseases. Following injection, the viral vectors will circulate until they recognize host cells with the appropriate target specificity for infection. Alternatively, the method as set forth by Tuomanen et al. (1993) can be used.

The vectors can be introduced into cells or tissues by any one of a variety of known methods

within the art. Such methods can be found described in Sambrook et al. and Ausubel et al., and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. Introduction of nucleic acids by infection offers several advantages over the other listed methods as indicated herein. Higher efficiency can be obtained due to their infectious nature. Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the vectors to specific cell types *in vivo* or within a tissue or mixed culture of cells. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

An alternate mode of administration of the vector can be by direct inoculation locally at the site of the disease or pathological condition or by inoculation into the vascular system supplying the site with nutrients. Local administration is advantageous because there is no dilution effect and, therefore, a smaller dose is required to achieve expression in a majority of the targeted cells. Additionally, local inoculation can alleviate the targeting requirement required with other forms of administration since a vector can be used that infects all cells in the inoculated area. If expression is desired in only a specific subset of cells within the inoculated area, then promoter and regulatory elements that are specific for the desired subset can be used to accomplish this goal. Such non-targeting vectors can be, for example, viral vectors, viral genome, plasmids, phagemids and the like. Transfection vehicles such as liposomes can also be used to introduce the non-viral vectors described above into

recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

The expression vector of the present invention may be administered to the patient alone or in combination with liposomes or other delivery molecules. The expression vector is administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, and other factors known to medical practitioners. The "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve in the treated patients a reduction in protein aggregation and may also include but is not limited to improved survival rate, more rapid recovery, or improvement or elimination of symptoms and are selected as appropriate measures by those skilled in the art.

While it is possible for the expression vector containing the sequence for the anti-aggregation molecule to be administered alone, it is preferable to present it as a pharmaceutical formulation. The formulations of the present invention comprise at least one active ingredient: the monoclonal antibody or expression vector together with one or more pharmaceutically acceptable carriers and optionally other therapeutic ingredients. The carrier(s) must be acceptable in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipients. The carriers must also be selected so as not to interfere with the activity of the active ingredient.

Further the present invention also provides a method of improving solubility and yields in

production of recombinant proteins, particularly in blocking the formation of inclusion bodies in the host cells for the *in vitro* production of recombinant proteins. The present invention provides an anti-aggregation molecule that suppresses the kinetics of aggregate formation while still encouraging formation of native protein structure, and favors the desired folding reaction thereby improving yields of the recombinant product. A genetically engineered antibody fragment as described herein can be used or a small peptide which mimics the antigen binding site on the target molecule can be used.

In a preferred embodiment a single-chain antibody (single-chain variable region; SCFVS) in which the heavy and light chain variable domains is engineered as a single polypeptide (Dueñas et al., 1994; Marasco et al., 1993) and delivered to the cells, either directly by methods described herein above or in an appropriate expression vector as described herein. Utilizing an expression vector, the expression of the antibody and of the desired gene product, in general an overexpressed recombinant product, are effected by introducing the either two vectors one encoding the gene for the recombinant produce and one for the antibody or a vector containing both into the host cell. The result is a small, approximately 28 KDa molecule with high-affinity ligand-binding capability and minimal assembly requirement that is co-synthesized with the recombinant protein product in the host cell. The antibody can be directed to the relevant cellular compartment using classical intracellular-trafficking signals. Functional studies have illustrated that single chain antibodies are able to fold and assemble correctly in the cytoplasm. The co-expression of the antibody and the recombinant product increases

intracellular production of the fusion proteins and prevents the formation of the inclusion bodies and/or facilitates extracellular protein secretion. The fusion proteins obtained, in addition to better solubility and yield, exhibit increased thermal and proteolytic resistance.

The availability of monoclonal antibodies which bind to a specific antigen at distinct and well defined sites has led to a better understanding of the effects of highly specific enzyme-antibody interactions on the enzyme behavior. By appropriate selection it has been possible to isolate those antibodies that are non-inhibitory to biological activity of the enzyme and bind at "strategic locations" on the antigen molecule, resulting in a considerable stabilization effect of the enzyme conformation. Moreover, such monoclonal antibodies, by selection using the present invention, prove to have a chaperone-like activity leading to a considerable refolding effect on the enzyme which was already partially heat denatured. In addition, the use of engineered monoclonal antibodies and their fragments, as well as peptides which mimic the binding site for the antigen on the antibody can be used in the present invention.

In the model test system shown in Example 1 herein below, Carboxypeptidase A (CPA) shows a decrease in solubility with an increase in temperature, accompanied by loss of enzymic activity and conformational changes leading to its aggregation. In the Example, the suppression of enzyme aggregation via its interaction with two monoclonal antibodies raised against native protein was investigated. ELISA measurements and determination of residual enzymic activity, as a probe of the native structure, were used to monitor the protein aggregation process. The

studied monoclonal antibodies are non-inhibitory to the biological activity of the antigen or target molecule, bind on the strategic position on the molecule and proved to have a chaperone-like activity in the prevention of protein aggregation. The antibodies effect on the inhibition of aggregation was found to be related to the location of the antigenic site of each antibody. Based on the experimental data, the formation of the immunocomplexes will provide a general and convenient method for suppression of aggregation and stabilization of the target molecules without affecting the biological properties of the given target molecule. The present invention uses genetically engineered antibodies obtained from such selected antibodies as protecting agents of *in vivo* aggregation of their antigen, leading to production of a soluble and stabilized protein.

Protein aggregation is of major importance that extends into mechanisms of human diseases and fundamental aspects of protein folding, expression and function. Data in the literature (De Young et al., 1993; Wetzel, 1994; Wetzel, 1991) suggests that aggregation is non-specific in the sense that addition of other proteins can influence the extent of aggregation of a certain protein. However, the specificity can be related to a particular residue or group of residues which play a special role in the folding-related aggregation of a polypeptide (Silen and Agard, 1989; Zhu et al. 1989; Winter et al., 1994; Brems 1988). The identification of such classes of sequences that play a role in the folding-unfolding and/or solubilization-aggregation provides the basis of the present invention for the prevention of aggregation.

Stabilization procedures based on protein-protein recognition processes, fundamental to biology, have been previously investigated (Chothia and Janin, 1975; Jaenicke, 1991). Introduction of molecular chaperones which enable folding and stabilization of unrelated proteins appears to be tailored to prevent misfolding and aggregation at an early stage during folding. However, the central problem remaining in *in vivo* folding is how to efficiently prevent aggregation without blocking the forward pathway of correct folding and biological activity of the native state (Ellis et al. 1991; Gething and Sambrook, 1992; Hendrick and Hartl, 1993).

The availability of monoclonal antibodies (mAbs) led to a better understanding of the effect of highly specific antigen-antibody interactions on the antigen or target molecule behavior. The complementary conformation between the interacting regions of the antibody with its antigen confers the high specificity and stability to the immunocomplex formed (Goldberg, 1991). Properly selected monoclonal antibodies, unlike the ubiquitous nature of the chaperones, bind to a specific antigen at a distinct and preselected antigenic site without interfering in the biological activity of the antigen and assist in antigen refolding (Blond and Goldberg, 1987; Carslon and Yarmush, 1992; Solomon and Schwartz, 1995).

The present invention utilized the effect of immunocomplexation in the suppression of antigen aggregation using as a model system the interaction of Carboxypeptidase A (CPA) and its monoclonal antibodies. CPA occupies a prominent position in the literature of metalloenzymes, being a well-characterized zinc exopeptidase that exhibits both peptidase and esterase activity (Vallee and Galdes, 1984). A large number of mAbs were prepared by the

applicant towards native enzymes (Solomon et al. 1984) and their properties were investigated. Some of these antibodies bind to the enzyme with a relatively high binding constant, remote from its active site and assist in refolding of already heat denatured enzyme (Solomon and Schwartz, 1995). ELISA measurements and determination of residual enzymic activity as a probe of native structure are used to monitor the effect of two different mAbs, namely CP_{10} and CP_9 on the inhibition of CPA aggregation.

Further, as shown in Examples 2 and 3 herein below, in suppressing β-amyloid aggregation, the monoclonal antibodies AMY-33 and 6F/3D, which recognized different epitopes of the β-amyloid peptide chain, exhibited a selective chaperone-like activity. The immunocomplex of mAb AMY-33 + βA4 not only prevented self-aggregation of β-amyloid peptide but also the aggregation that was induced in the presence of heparan sulfate, which is thought to affect only the aggregation of preexisting amyloid fibers (Talafous et al., 1994). The inhibitory effect was related to the localization of the antibody-binding sites and to the nature of the aggregating agents. The results of negative-staining electron microscopy revealed that even at low concentrations of mAb AMY-33 only amorphous aggregates are formed. The ELISA measurements indicated that increasing the concentration of mAb AMY-33 to equimolar antigen/antibody ratios maintained β-amyloid peptide solubility. The diffuse and amorphous conglomerates of Aβ deposits that were not detectable by thioflavin T fluorimetry or Congo Red staining are not supposed to be associated with neuritic pathology (Levine, 1993).

Because β-amyloid peptide has been shown to be physiologically produced in a soluble form in

normal individuals (Seubert et al., 1992; Shoji et al., 1992), the aggregation of soluble β-amyloid peptide into insoluble amyloid fibrils is believed to be a crucial step in the pathogenesis of Alzheimer's disease. Therefore, to reduce or eliminate the extent of pathological protein depositions in the brain, much effort has been focused on developing potent and selective inhibitors of β-amyloid aggregation (Synder et al., 1994; Tomiyama et al. 1994; Schwarzman et al., 1994). Preparing mAbs against "aggregating epitopes," identified as sequences related to the sites where protein aggregation is initiated, thereby provides a tool for preventing the phenomenon of protein aggregation. Applicant has shown that appropriate mAbs interact at strategic protein-folding-initiation sites, leading to a considerable refolding effect of the already clustered epitopes.

The mAb AMY-33 did not exhibit a similar inhibitory effect on metal-induced amyloid aggregation. The slight interference with Zn^{2+}-induced β-amyloid peptide aggregation that occurred using mAb 6F/3D may be due to the partial solvation effect of already aggregated β-peptide.

In experiments with additional monoclonal antibodies, mAbs 6C6 and 10D5, which recognize an epitope spanning the amino acid residues 1-16 of β-amyloid peptide, inhibited the formation of β-amyloid by 90% when compared with aggregation occurring in the absence of the respective antibodies. mAb AMY-33, which recognizes another epitope located within residues 1-28, affected the self-aggregation of β-peptide to a lower extent of approximately 40% in the same set of experiments. The antibodies, 2H3 and 1C2, directed to the regions comprising peptides 1-12 and 13-28, respectively, had a considerably lower effect on *in vitro* amyloid formation.

The above discussion provides a factual basis for the use of monoclonal antibodies and genetically engineered antibody fragments as therapeutics for the prevention of protein aggregation. The methods used with and the utility of the present invention can be shown by the following examples.

EXAMPLES

METHODS AND REAGENTS

Carboxypeptidase A (CPA)

CPA was obtained as an aqueous crystalline suspension (Sigma Chemical Co., St. Louis, MO). The crystals were washed with double-distilled water, centrifuged, and dissolved in 0.05 M Tris-HCl/0.5 M NaCl buffer, pH 7.5. Insoluble material was removed by centrifugation. The enzyme concentration was derived from the absorbance at 278 nm.

Determination of CPA Enzymatic Activity

The enzymatic activities of CPA and its immunocomplexes were determined spectrophotometrically at 254 nm using either 1 mM hippuryl-L-phenylalanine as peptidase substrate or hippuryl-DL-β-phenyllactic acid as esterase substrate in 0.5 M NaCl/0.05 M Tris-Hcl, pH 7.5, (Solomon et al, 1989).

Amyloid

Amyloid peptides, Aβ 1-40 (Cat. No. A-5813) and Aβ 1-28 (Cat. No. A-1084) corresponding to amino acids 1-40 and 1-28 of Aβ respectively, were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Amyloid solutions were prepared by dissolving the peptides in water at concentration of

10 mg/ml. The stock solution was stored in aliquots at -20°C.

Aggregating agents

Heparan sulfate (Cat. No. H 5393) was purchased from Sigma Chemical Co., St. Louis, MO, USA. Stock solutions of metal chlorides were made up from dry salts at concentration of 1mM in TRIS pH 7.4.

Monoclonal Antibody Production

In general, monoclonal antibodies may be prepared against a synthetic peptide based on the sequence, or prepared recombinantly by cloning techniques or the natural gene product and/or portions thereof may be isolated and used as the immunogen. Such proteins or peptides can be used to produce monoclonals by standard production technology well known to those skilled in the art as further described generally in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988, Milstein (1980) and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.M. Freeman and Co., 1992. Briefly, mouse monoclonal antibodies are prepared by hyperimmunization of an appropriate donor with the protein or peptide fragment, generally a mouse, and isolation of splenic antibody producing cells. These cells are fused to a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid which has immortality and secretes the required antibody. The cells are then cultured, in bulk, and the monoclonal antibodies harvested from the culture media for use.

The harvested monoclonal antibody can be bound to a solid support substrate or conjugated with a detectable moiety or be both bound and conjugated as is well known in the art. For a general discussion of

conjugation of fluorescent or enzymatic moieties see Johnstone & Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, Oxford, 1982. The binding of antibodies to a solid support substrate is also well known in the art. (see for a general discussion Harlow & Lane *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Publications, New York, 1988) The detectable moieties contemplated with the present invention can include, but are not limited to, fluorescent, metallic, enzymatic and radioactive markers such as biotin, gold, ferritin, alkaline phosphatase, β-galactosidase, peroxidase, urease, fluorescein, rhodamine, tritium, ^{14}C and iodination.

Alternatively, commercially available antibodies can be used. α-Human β-amyloid 6F/3D was obtained from ACCURATE Chemical and Scientific Corp. (Westbury, NY, USA). mAb AMY 33 was purchased from ZYMED San Francisco, CA, USA. A polyclonal, affinity purified rabbit IgG obtained against the synthetic Alzheimer β-amyloid (Cat. No. 1381431) was purchased from Boehringer-Mannheim, GmbH, Germany. Another four monoclonal antibodies called 6C6 and 10D5, raised against peptide 1-28 of β-amyloid, 2H3 and 1C2 raised against peptides 1-12 and 13-28, respectively, were generously provided by Dr. D. Schenk, Athena Neuroscience, San Francisco, CA.

Purification and characterization of anti-CPA mAbs

The monoclonal antibodies, CP-10, CP-9, which interact with CPA at high binding constants, were selected for further study. The preparation and characterization of the monoclonal antibodies CP_{10} and CP_9 (chosen for the present study) were previously described (Solomon et al., 1989; Solomon and Balas, 1991).

These antibodies were isolated and purified by affinity chromatography on protein A-Sepharose from the corresponding ascites fluids according to Harlow and Lane.

Protocol for determining effect of monoclonal antibody binding on CPA activity

CPA (1 mg/ml) was incubated at 50°C in the absence and in the presence of increasing amounts of mAbs CP_{10} and CP_9 (100 μl in PBS) ranged between 0-2 molar ratio antibody/CPA. The enzymic activities of the immunocomplexes formed were measured as described herein above. Data related in percentage, 100% being considered the enzymic activity of CPA before denaturation.

ELISA Tests for CPA Studies

The antigen-coating solutions (100 μl containing native CPA (10-25μl/ml) in PBS, pH 7.4, were incubated overnight at 4°C in a polystyrene ELISA plate (Costar, Cambridge, MA). Diluted ascites fluid (0.1 ml) containing the desired mAb (1:2000 to 1:18,000 v/v in PBS) was added and incubated at 37°C for 1 hour. The amount of bound mAb was determined with β-galactosidase-linked $F(ab)_2$ fragments of sheep anti-mouse IgG (Amersham International, UK).

The quantitation of the amount of aggregated CPA during denaturation at 50°C was determined by competitive and sandwich ELISA, as follows:

Competitive ELISA assays for CPA Studies

CPA (10 μl/ml of PBS) was adsorbed onto ELISA plates overnight at 4°C, the remaining active groups on the plate being blocked with non-fat milk. To the soluble CPA (200 ng in 10 μl PBS), incubated for one hour at 50°C, the mAb CP_{10} (molar ratio 1:1

Ab/CPA) was added and allowed to interact with the remaining soluble CPA for one hour at 37°C. In parallel, the mAb was added to the CPA solutions before exposure at 50°C for one hour. After incubation, the CPA preparations were removed by centrifugation at 15,000 rpm for 15 minutes and applied on the ELISA plates coated with CPA. The antibody which did not bind to soluble CPA in the reaction mixture will bind to the coated CPA; the amount of antibody bound to the coated antigen will be conversely proportional to the extent of CPA aggregation and determined using α-mouse antibodies labeled with horseradish peroxidase (HRP). The color developed by HRP (*O*-phenylenediamine (OPD) as substrate) was measured at OD_{495} using an ELISA plate reader. The amount of antibody bound on the coated CPA in the absence of soluble CPA was considered as 100%.

Sandwich ELISA for CPA Studies

The ELISA plates were coated with rabbit polyclonal antibodies raised against CPA (1μl/well) by incubation at 37°C for two hours. The residual active groups were blocked by non-fat milk. Soluble CPA (200 ng in 10μl PBS) was exposed to 50°C for one hour and the aggregated CPA was removed by centrifugation at 15,000 g for 15 minutes. The residual soluble CPA was incubated for another one hour at 37°C with mAb CP_{10} and mAb CP_9 at various molar ratio antibody/antigen. In another set of experiments, the mAbs were added to the reaction mixtures before incubation at 50°C and then exposed for one hour at 50°C. After the incubation period, all the immunocomplexed CPA preparations were centrifuged and added to the ELISA plate, previously coated with polyclonal CPA antibodies, for 12 hours at 4°C. The amount of mAb

bound, determined as described above, will be proportional to the amount of soluble CPA which remained after exposure to aggregation conditions. The results are presented in percentages, 100% being the maximal absorbance obtained before CPA heat treatment.

All data presented are the mean of triplicate determinations. The standard deviation of the intra-assay and interassays were less than 5% in all cases.

Amyloid ELISA Assays

The ELISA plates were coated with rabbit polyclonal antibodies (Boeringer-Mannheim) raised against synthetic β-amyloid (1-40) (Sigma) (100 ng/well) via covalent attachment to epoxy-coated ELISA plates by incubation at 4°C for 16 hours. (Eupergit-C containing epoxy groups, Rohm GmbH, Darmstadt, Germany, using beads or paper as the solid phase Solomon, et al., 1992; Solomon et al., 1993). The residual epoxy groups were blocked by non-fat milk.

The reaction mixtures containing aqueous solution of β-amyloid (100 ng/ml), heparan sulfate (50 mM) and/or chloride metal solutions (10^{-3}M at pH 6.5), was first incubated at 37°C for three hours. The aggregated β-amyloid preparations were removed by centrifugation at 15,000 g for 15 minutes. The residual soluble β-amyloid was incubated for another one hour at 37°C with mAbs AMY 33 and/or 6F3D at equal molar ratio antibody/antigen. In another set of experiments, the mAbs were added to the reaction mixtures before incubation at 37°C and then incubated together for 3 hours at 37°C.

After the incubation period, the immunocomplexed amyloid preparations were added to the ELISA plates, previously coated with polyclonal anti-

amyloid antibodies. The amount of mAb bound will be proportional to the amount of soluble amyloid which remained after exposure to aggregation conditions.

The amount of bound antibody was determined using α-mouse second antibodies labeled with horseradish peroxidase (HRP). The enzyme activity of HRP is directly proportional with the amount of residual amyloid bound to rabbit polyclonal antibodies. The enzyme activity of HRP was measured using *O*-phenylenediamine (OPD) as substrate. The color developed was measured at A_{495} using an ELISA reader. Data represent the mean of triplicate determinations. The standard deviation of the intra-assay and interassays were less than 5% in all cases.

Competitive enzyme-linked immunosorbent assay (ELISA)

β-Amyloid peptide (50 ng/well in PBS) was covalently bound to epoxy-coated ELISA plates overnight at 4°C. The remaining active groups on the plate were blocked with skim milk.

The reaction mixture containing soluble β-amyloid peptide (50 ng in 10 μl PBS) was first incubated for three hours at 37°C. Each of the mAbs was added and allowed to interact with the remaining soluble β-amyloid peptide for one hour at 37°C. The amount of each antibody was sufficient to bind to all soluble β-amyloid peptide (50 ng) before first incubation at 37°C. In parallel assays, the same amounts of mAbs were added to the β-amyloid peptide solutions before the first incubation at 37°C for three hours. After the second incubation, the β-amyloid preparations centrifuged at 15,000 x g for 15 minutes and the soluble reaction mixture was applied onto the ELISA plates previously coated with β-amyloid peptide.

The antibody that does not bind to the insoluble β-peptide removed by centrifugation will bind to the β-amyloid peptide coated on the ELISA plate. The amount of antibody bound to the β-amyloid peptide coated plate will be inversely proportional to the amount of residual soluble β-amyloid peptide. The amount of bound antibody was detected using α-mouse antibodies labeled with horseradish peroxidase (HRP). Degradation of the *O*-phenylenediamine (OPD) substrate by HRP was monitored at OD_{495} using an ELISA reader. The maximal amount of antibody necessary to bind all the coated β-amyloid peptide in the absence of β-soluble peptide was determined for each mAb and was considered as 100% of mAb bound. The data represent the mean of five replicates.

β-Amyloid Peptide Aggregation and Immunocomplexation

Synthetic β-amyloid peptide 1-40 (Aβ 1-40) was obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Reaction mixture tubes containing 200 µl of an aqueous solution of β-amyloid peptide (10 mg/ml) pH 6.7 were incubated for three hours at 37°C. Aggregated β-amyloid samples were removed by centrifugation at 15,000 x g for 15 minutes. Aliquots of residual soluble β-amyloid peptide were then incubated for another 60 minutes with increasing amounts of the appropriate mAbs to produce totally immunocomplexed β-amyloid peptide. In another set of experiments, mAbs at equimolar antibody/antigen concentrations were added to the reaction mixtures before the first incubation period of three hours at 37°C.

Electron Microscopy.

Negatively stained amyloid fibrils were prepared by floating carbon-coated grids on aqueous

peptide solutions (1-2 mg/ml) and air drying. Fibrils of β-amyloid, either alone or immunocomplexed to mAb AMY-33 (molar ratio 4:1) for three hours at 37°C, were negatively stained with aqueous uranyl acetate (2% wt/vol) and then visualized using a JEOL-1200 Ex electron microscope operated at 80/KV, using a magnification of 25,000.

Fluorimetry.

Fluorimetric analysis of soluble β-amyloid peptide and the immunocomplex with AMY 33 (molar ratio 4:1) stained with Thioflavin T (Sigma Chemical Co., St. Louis, MO., USA) was performed by standard method (Levine, 1993). Fluorescence was measured using a Perkin-Elmer LS-50 fluorimeter at λ_{ex} = 482 nm. The aggregation reaction was followed for seven days at 37°C.

EXAMPLE 1

CPA Model System

Aggregation of heat denatured CPA was followed by determination of the residual enzymic activity of CPA using esterase and peptidase substrates. CPA (1 mg/ml) was incubated at various temperatures for one hour, and residual enzymic activity was determined. The temperature of 50°C was chosen for further study. At this temperature, mAbs studied keep all their immunological activity (unpublished data). Effect of immunocomplexation of CPA with its mAbs was monitored by: (1) Determination of enzymic activity and (2) ELISA measurements as described herein above.

Monoclonal antibodies raised against native antigens proved to be powerful tools in identification and characterization of folding steps by recognition of incompletely folded antigens (Hendrick and Hartl,

1993). The selected antibodies might interact at sites where protein unfolding is initiated, thereby stabilizing the protein and suppressing further aggregation.

The main difference between mAbs and molecular chaperones is that the latter does not bind to native proteins and is capable of interacting with many different polypeptide chains without exhibiting an apparent sequence preference (Goloubinof et al., 1989). Moreover, chaperones suppress aggregation but do not redissolve aggregate proteins already present.

The aggregation of CPA and loss of its enzymic activity was found to be dependent on the temperature and the time of incubation (Figures 1, 2). Esterase activity seems to be more affected at higher temperature than peptidase activity, indicating that these activities follow different reaction mechanisms (Figure 2). These data are compatible with applicant's previous results (Solomon et al., 1989; Solomon and Balas, 1991), as well as with the findings of Vallee and his collaborators (1969), who postulated that the active site of CPA consists of non-identical but interacting binding sites for peptides and ester substrates. As shown in Figure 2, the immunological recognition of partially heat denatured enzyme is better conserved than its residual enzymic activity.

The inhibition of CPA aggregation, induced by incubation at 50°C for one hour by its interaction with two mAbs, CP_9 and CP_{10}, was followed by measuring the peptidase and esterase enzymic activities (Figure 3). The two mAbs, CP_{10} and CP_9 were chosen for this study on the basis of previous data regarding their effect on the enzyme behavior (Solomon and Schwartz, 1995; Solomon et al., 1989; Solomon and Balas, 1991). The protection of enzymic activity of heated CPA was dependent on the amount of antibody added to the

enzyme and a molar ratio of 1:1 antibody/enzyme was sufficient for the maximum protection effect. The peptidase activity of the CPA-CP_{10} complex was maintained at 90% of its initial activity in the presence of mAb CP_{10}. The protective effect of mAbs on CPA activity during heat denaturation was found to be related to the location of the antigenic site of each antibody (Figure 4). Even a great excess of unrelated antibody did not assist in maintaining CPA activity. Increase in preservation of enzyme activity can be reached, however, in the presence of a pair of two antibodies. This effect seems to be the result of a "locking" of the conformation caused by simultaneous interaction with two different antibodies at two distinct epitopes (Solomon and Balas, 1991).

The amount of aggregated CPA was quantitated by ELISA measurements. Disappearance of CPA, as a result of its aggregation during incubation for one hour at 50°C, was followed by a competitive ELISA assay (Figure 5) and a sandwich assay (Figure 6). The mAb, CP_{10}, maintained 100% of the CPA activity in solution during heating for one hour at 50°C (Figure 6); CP_9 provided a slight effect on CPA protection at 50°C. Both antibodies prevent the aggregation of CPA, similar to the data shown in Figure 4, recognizing "key positions" on the molecule responsible for heat denaturation and aggregation of CPA.

The biological activity of the enzyme seems to be more sensitive to high temperatures than the insolubilization process. Subtle heat-induced conformational changes occurring in CPA molecules are reflected by change in enzymic activity, even before transition between native-molten globule conformation-aggregated states occurred. These findings are in contradiction to previous suggestions that the

biological function of a protein does not necessarily require fully folded protein (Hattori et al., 1993).

The antigen binding site of mAb CP_{10} (previously named CP_{100}) was identified as one of the immunodominant regions of the enzyme, localized on the surface of the molecule between amino acids 209-218 (Solomon et al. 1989). The localization of the epitope recognized by CP_9 has not yet been clarified, but it does not interfere with the mAb CP_{10} during simultaneous binding to CPA molecule, as suggested by additivity measurements (Solomon and Balas, 1991).

Similar effects in suppression of antigen aggregation were obtained after immunocomplexation of horseradish peroxidase.

The data available in the literature of antibody preparation suggests that for practically all antigens it should be possible to prepare monoclonal antibodies and by means of the present invention it will be possible to select those monoclonal antibodies which bind with high affinity without affecting the target molecule activity. Moreover, mAbs like the majority of immunoglobulins, are robust molecules and survive in a variety of environments, including high temperatures, low pH, denaturing agents. Formation of such immunocomplexes should provide a general and convenient method for suppression of aggregation and stabilization of their antigen without affecting the biological properties of the given antigen as shown also in the following Examples.

EXAMPLE 2

Amyloid System

This example investigates the immunocomplexation effect on the *in vitro* aggregation of β-amyloid. Aggregation of β-amyloid was found to be dependent on the pH, peptide concentration,

temperature and time of incubation (Burdick et al., 1992). In applicant's experiments, the aggregation of β-amyloid was performed by incubation of an aqueous solution of βA4 (10 mg/ml) for three hours at 37°C. The β-amyloid aggregation was followed by ELISA measurements using two different commercially available monoclonal antibodies raised against β-amyloid: α-human β-amyloid 6F/3D obtained from Accurate Chemical and Scientific Corp, Westbury, N.J. USA, and mAb AMY 33 (Stern et al., 1990), purchased from Zymed, San Francisco, CA, USA, raised against peptides 8-17 and 1-28, respectively, of the β-amyloid.

The addition of the antibodies was made before or after exposure of synthetic β-amyloid to the aggregation process (Figs. 7A, B). The aggregation of the β-amyloid was performed in the presence of heparan sulfate and/or metal ions, such as Zn^{2+} and Al^{3+}. The antibody AMY-33, which recognizes an epitope spanning the amino acids 1-28 of the β-amyloid sequence, inhibits the β-amyloid aggregation occurring in the presence or absence of heparan sulfate (Figure 7A). Any significant effect on metal-induced amyloid aggregation was observed under the same experimental conditions. The mAb 6F/3D, recognizing an epitope located in amino acids 8-17 of β-amyloid, interferes with Zn^{2+}-induced aggregation, showing a partial solubilization effect on already aggregated β-amyloid, but has no effect on other aggregating agents (Figure 7B).

Metals, such as Zn^{2+} and Al^{3+}, have been proposed as risk factors for Alzheimer's disease development (Mantyh et al., 1993; Frederickson, 1989; McLachlan et al., 1991). The aggregation of βA4 induced by aluminum is distinguishable from that induced by Zn in terms of role, extent, pH and

temperature dependence (Mantyh et al. 1993). Although the precise site of interaction of metal ions and βA4 is not clarified, several residues in βA4 are candidates for metal binding. The βA4 histidine residues (His_{13}-His_{14}) may be implicated in fibril formation and it is conceivable that at least H_{14} remains available for intermolecular electrostatic interactions between anti-parallel chains (Talfous et al., 1994). The site defined by Val_{12}-His_{13}-His_{14}-Glu_{15}-Lys_{16}-Leu_{17} has been identified as a sequence containing a heparan sulfate binding domain (Fraser et al., 1992) and His_{13} and Lys_{16} are supposed to provide the cationic binding sites being exposed on the same face of the peptide β sheet (Talafous et al. 1994).

Binding of mAb AMY-33 to βA4 prevents self-aggregation of the β-amyloid. This antibody prevents intramolecular aggregation occurring in the presence of heparan sulfate, which is supposed to affect only the aggregation of preexisting amyloid fibers (Fraser et al., 1992). Inhibition of β-amyloid aggregation in the presence of mAb 6F/3D was partially effective only in the presence of Zn^{2+}.

EXAMPLE 3

Amyloid Electron Microscopy and Fluorimetric Studies

Electron microscopy of negatively stained β-amyloid and its immunocomplex with mAb AMY-33 revealed that even at a low peptide/antibody ratio, fibrillar β-amyloid was converted to an amorphous state. Thioflavin T, a suitable probe for detecting the fibrillar aggregation of β-amyloid peptide, confirmed the electron microscopy results. Dilution of the peptide directly from water into dye-containing buffer had no effect on dye fluorescence. In the presence of aggregated Aβ4 (1-40), however, a change occurred in the excitation spectrum of Thioflavin T,

manifested as a new peak at 450 nm that was not seen with the free dye (Figs 8A, B.) Aggregated β-amyloid peptide enhances the fluorescence emission of Thioflavin T at 482 nm as a function of incubation-time. Adding mAb AMY-33 (4:1) to the Aβ 1-40 solution before exposure to 37°C prevented the increase in fluorescence at 482 nm, whereas the addition of unrelated antibody did not interfere with the fluorescence peaks.

EXAMPLE 4

Comparative Studies of Monoclonal Antibodies

Monoclonal antibodies were added to the reaction mixture before or after incubation of synthetic β-amyloid peptide at 37°C for three hours. The results shown in Figure 9 indicate that mAbs 6C6 and 10D5, which recognize an epitope spanning the amino acid residues 1-16 of β-amyloid peptide, inhibited the formation of β-amyloid by 90% when compared with aggregation occurring in the absence of the respective antibodies. The mAb AMY-33, which recognizes another epitope located within residues 1-28, affected the self-aggregation of β-peptide to a lower extent of approximately 40%. The antibodies, 2H3 and 1C2, directed to the regions comprising peptides 1-12 and 13-28, respectively, had a considerably lower effect on *in vitro* amyloid formation.

On the basis of applicants findings regarding these Examples and other antigen-antibody systems studies (Solomon et al., 1989; Solomon and Balas, 1991), the formation of the immunocomplexes with selected, highly specific monoclonal antibodies, should provide a general and convenient method to prevent aggregation of the proteins without affecting their biological properties.

At least 15 different polypeptides are known to be capable of causing *in vivo* different forms of amyloidosis via their deposition in particular organs or tissues as insoluble protein fibrils.

Recent advances in antibody engineering technology, as well as in the development of suitable delivery systems (Haber, 1992; Pluckthun, 1992; Travis, 1993; Marasco et al., 1993) make it possible to develop functional small antibody fragments to serve as therapeutic chaperones for the treatment of Alzheimer's disease as well as other human amyloidosis diseases by gene based therapies.

Application of the above findings for *in vivo* aggregation, can confer to single chain antibodies (Pluckthun, 1992) or other engineered antibody fragments, a protective role in the renaturation of recombinant proteins.

Throughout this application various publications are referenced by citation or number. Full citations for the publications referenced by number are listed below. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

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CLAIMS

What is claimed is:

1. A method of selecting anti-aggregation molecules with chaperone-like activity that have characteristics including binding to a native target molecule epitope with a high binding constant and are non-inhibitory to the biological activity of the target molecule by

denaturing a target molecule which aggregates,

mixing the target molecule with a presumptive anti-aggregation molecule,

incubating the mixture under conditions allowing for aggregation,

selecting non-aggregated mixtures, and

testing the nonaggregated target molecule coupled to the anti-aggregation molecule for bioactivity thereby selecting an anti-aggregation molecule with chaperone-like activity.

2. The method of claim 1 wherein the anti-aggregation molecule is a monoclonal antibody, a genetically engineered antibody fragment or a peptide which mimics the binding site for an antigen on the antibody.

3. The method of claim 1 wherein the anti-aggregation molecule is a single chain monoclonal antibody.

4. The method of claim 1 wherein the target molecule is β-amyloid.

5. The method of claim 1 wherein the target molecule is a prion protein.

6. A method of selecting anti-aggregation molecules with chaperone-like activity that have characteristics including binding to a target molecule epitope with a high binding constant, reversing aggregation effects and are non-inhibitory to the biological activity of the target molecule by

preparing an aggregated target molecule,

mixing the target molecule with a presumptive anti-aggregation molecule,

selecting mixtures with non-aggregated target molecules, and

testing the target molecule coupled to the anti-aggregation molecule for bioactivity thereby identifying an anti-aggregation molecule with chaperone-like activity.

7. The method of claim 6 wherein the anti-aggregation molecule is a monoclonal antibody, a genetically engineered antibody fragment or a peptide which mimics the binding site for an antigen on the antibody.

8. The method of claim 6 wherein the anti-aggregation molecule is a single chain monoclonal antibody.

9. The method of claim 6 wherein the target molecule is β-amyloid.

10. The method of claim 6 wherein the target molecule is a prion protein.

11. The method of treating a protein aggregation disease including the steps of

preparing at least one anti-aggregation molecule that binds to an aggregating protein which is the cause of a disease and which prevents aggregation while allowing bioactivity;

creating an expression vector comprising nucleic acid including a sequence which encodes in expressible form the anti-aggregation molecule that binds to an aggregating protein and which prevents aggregation while allowing bioactivity; and

administering the expression vector.

12. The method of claim 11 wherein the anti-aggregation molecule is a monoclonal antibody, a genetically engineered antibody fragment or a peptide which mimics the binding site for an antigen.

13. The method of claim 12 wherein the monoclonal antibody that binds to an aggregating protein and which prevents aggregation while allowing bioactivity is a single chain monoclonal antibody.

14. The method of claim 12 wherein the monoclonal antibody that binds to an aggregating protein and which prevents aggregation while allowing bioactivity is a human anti-β-amyloid monoclonal antibody.

15. The method of claim 12 wherein the monoclonal antibody that binds to an aggregating protein and which prevents aggregation while allowing bioactivity is an anti-prion protein monoclonal antibody.

16. The method of claim 11 wherein at least two anti-aggregation molecules are used.

17. The method of claim 11 wherein the expression vector is a virus.

18. The method of claim 17 wherein the expression vector is a disable retro virus.

19. The method of claim 17 wherein the expression vector is a retroviral shuttle vector.

20. The method of claim 17 wherein the expression vector is vaccinia virus.

21. The method of claim 17 wherein the expression vector is an adenovirus.

22. The method of claim 17 wherein the expression vector is a plasmid.

23. A pharmaceutical composition comprising an expression vector comprising nucleic acid including a sequence which encodes in expressible form an anti-aggregation molecule that binds to an aggregating protein while allowing bioactivity and a pharmaceutically acceptable carrier.

24. The expression vector as set forth in claim 23 wherein the anti-aggregation molecule is a monoclonal antibody, a genetically engineered antibody fragment or a peptide which mimics the binding site for an antigen.

25. The expression vector as set forth in claim 23 wherein the anti-aggregation molecule is a single chain monoclonal antibody.

26. The expression vector as set forth in claim 24 wherein the monoclonal antibody is a human anti-β-amyloid monoclonal antibody.

27. An expression vector for use as a therapeutic agent which comprises nucleic acid including at least one sequence which encodes in expressible form an anti-aggregation molecule that binds to an aggregating protein which is associated with a disease.

28. The method of treating a protein aggregation disease including the steps of

preparing at least one human monoclonal antibody that binds to an aggregating protein which is the cause of a disease and allows bioactivity when bound, and

administering the monoclonal antibody.

29. The method of claim 28 wherein the aggregating protein is β-amyloid.

30. The method of claim 29 wherein the monoclonal antibody is an anti-β-amyloid and is selected from the group consisting of AMY-33 which recognizes an epitope spanning amino acids 1-28 of β-amyloid and monoclonal antibodies 6C6 and 10D5, which recognize an epitope spanning the amino acid residues 1-16 of β-amyloid peptide.

31. A method of improving solubility and yields in production of recombinant proteins by

preparing an expression vector containing a genetic sequence for an anti-aggregation molecule that suppresses the kinetics of aggregate formation while still encouraging formation of native protein structure, and favors the desired folding reaction of a recombinant protein,

co-transforming a host cell with the expression vector for an anti-aggregation molecule and an expression vector for a recombinant protein thereby increasing intracellular production of the fusion proteins and preventing formation of inclusion bodies and facilitating extracellular protein secretion.

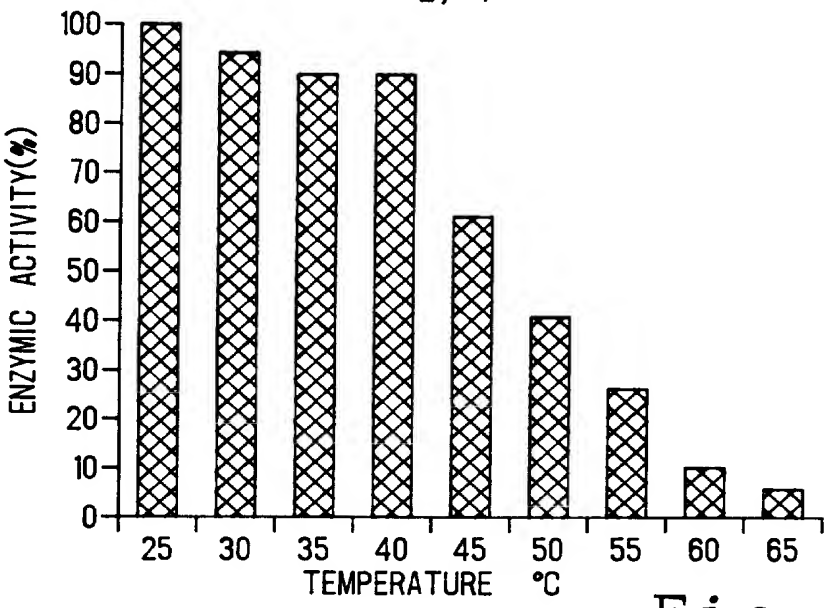

Fig-1

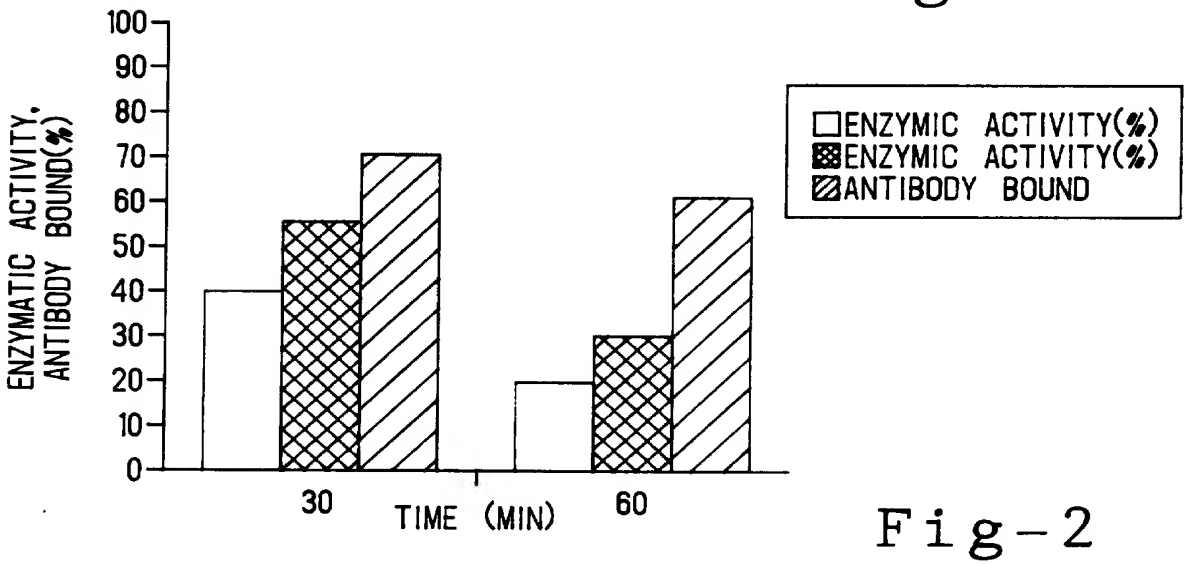

Fig-2

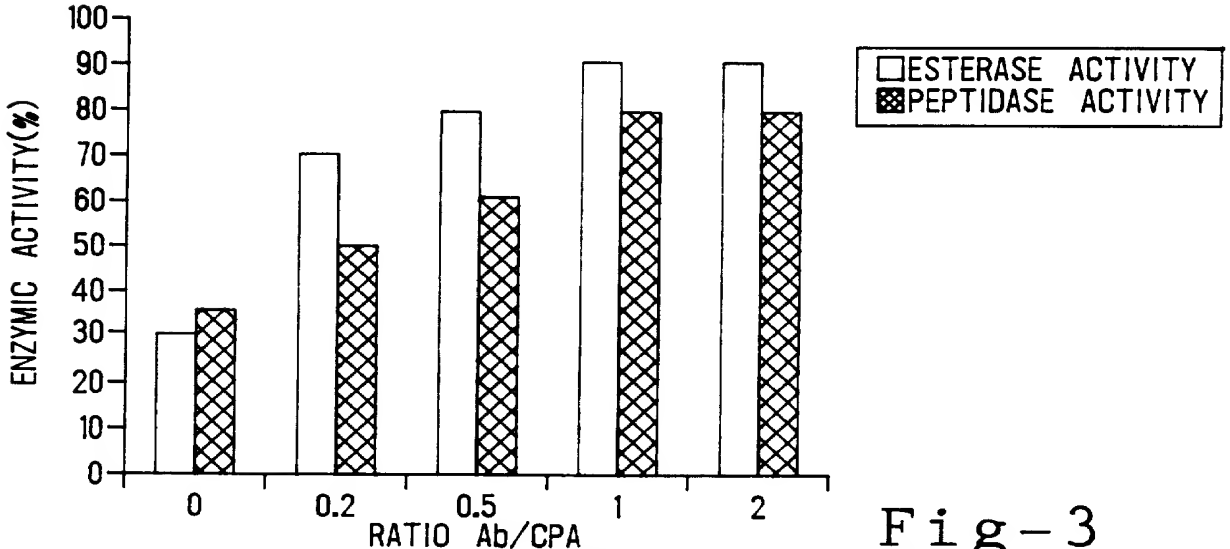

Fig-3

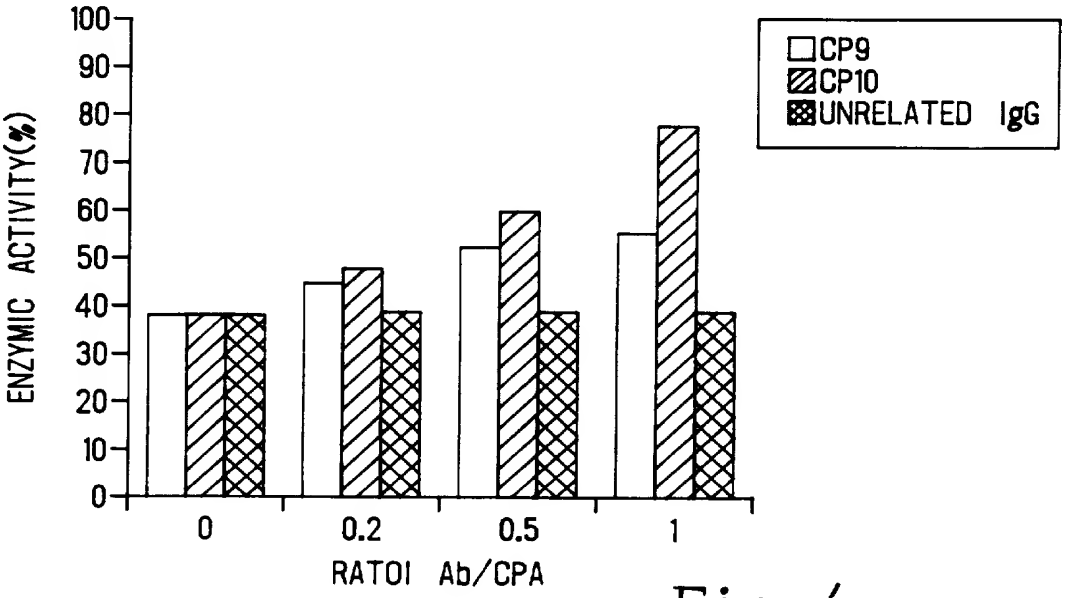

Fig-4

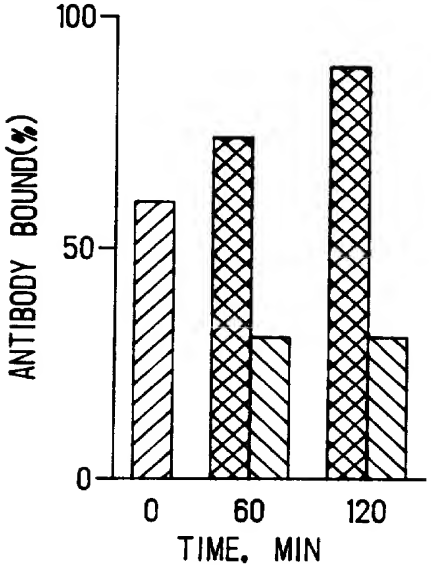

Fig-5

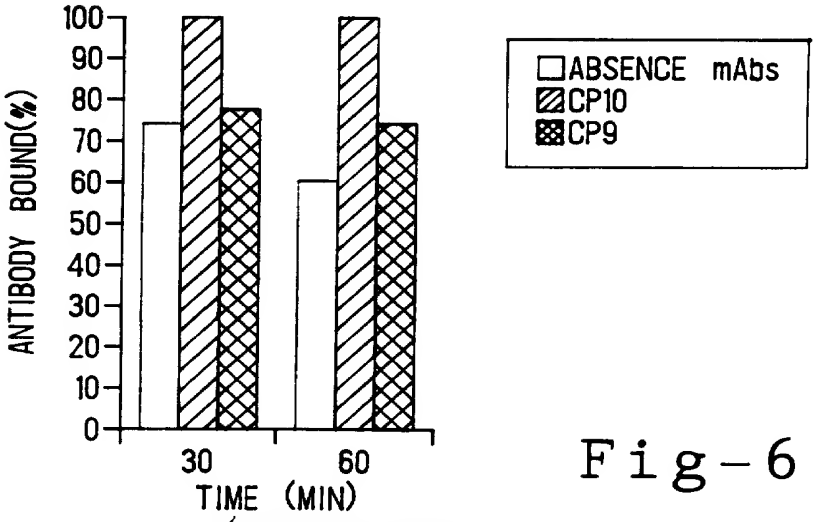

Fig-6

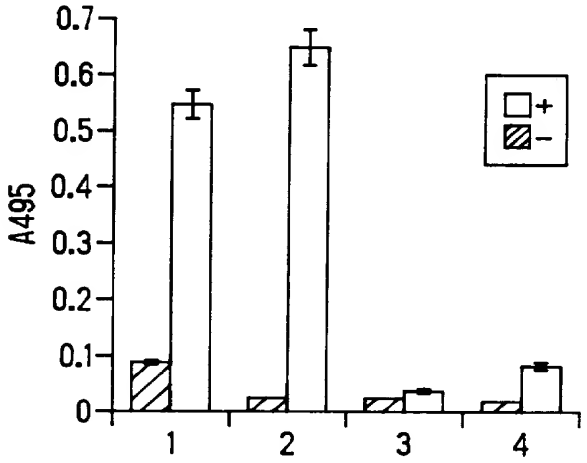

Fig-7A

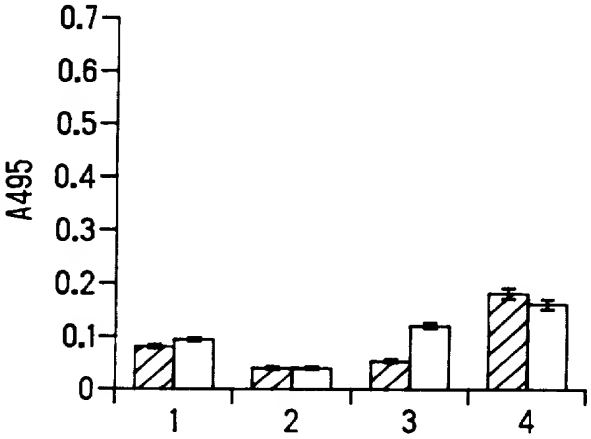

Fig-7B

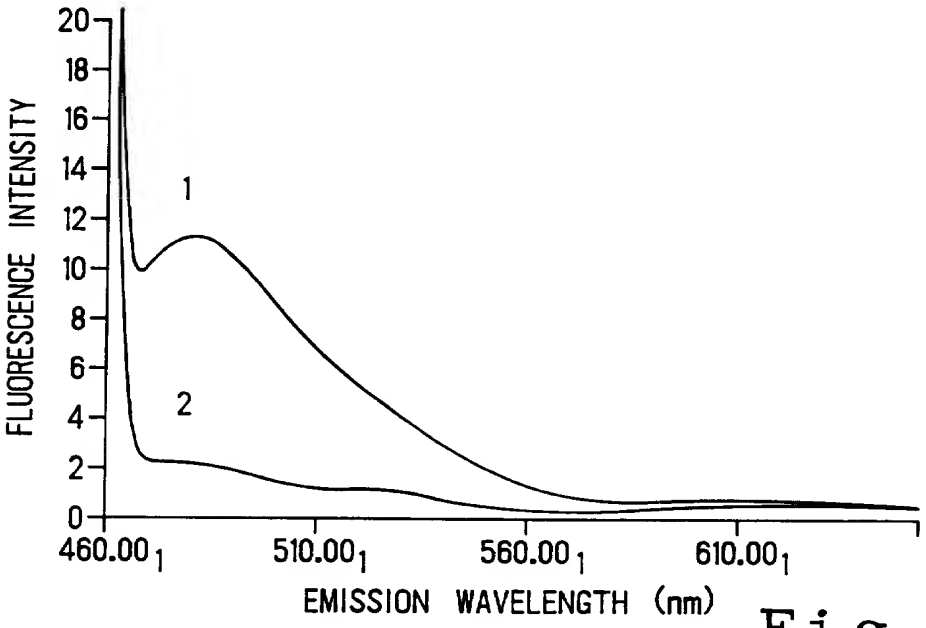

Fig-8A

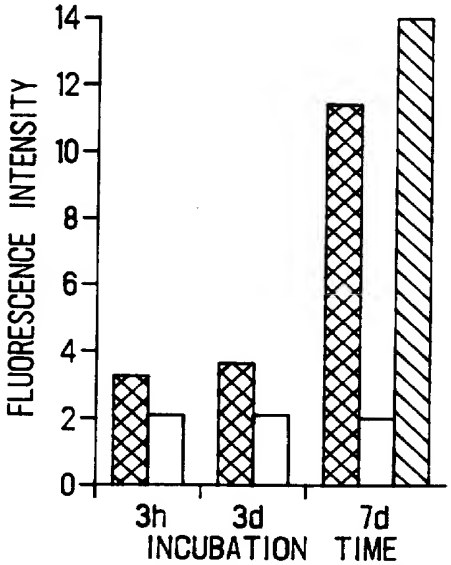

Fig-8B

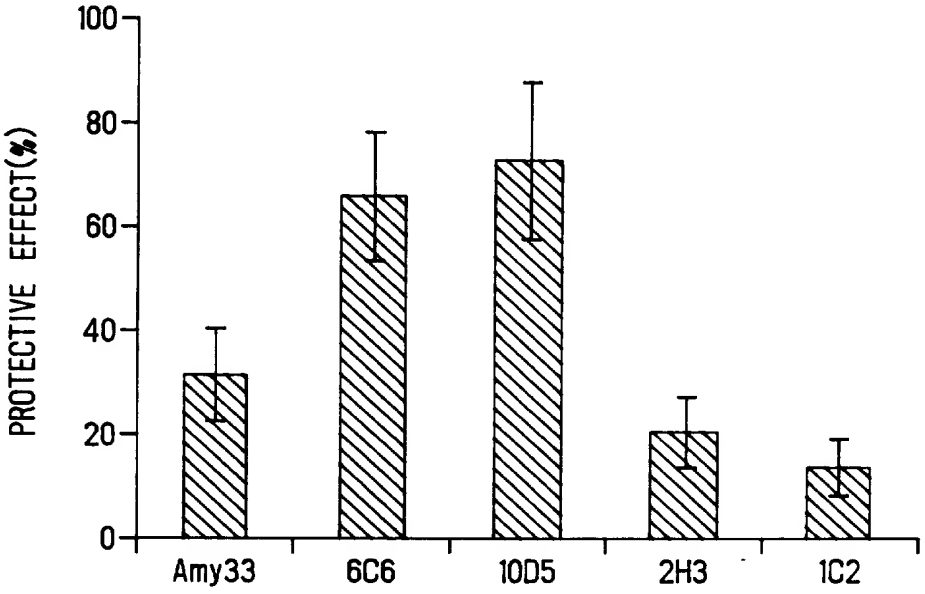

Fig-9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/16092

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/53; A61K 38/00, 31/66; C12N 15/00; C12P 21/06
US CL :435/7.1, 69.1, 320.1; 514/12, 130
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 69.1, 320.1; 514/12, 130

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CHEMICAL ABSTRACTS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Biochemical and Biophysical Research Communications, Volume 192, No. 2, issued 30 April 1993, Wisniewski et al, "Apolipoprotein E: Binding to Soluble Alzheimer's β-Amyloid", pages 359-365, especially pages 362-363.	1-30

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

03 APRIL 1996

Date of mailing of the international search report

23 APR 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/16092

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/16092

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s)1-10, drawn to a method of selecting anti-aggregation molecules.
Group II, claim(s) 11-21 and 27-29, drawn to a method of treating a protein aggregation disease.
Group III, claim(s) 22-26, drawn to a pharmaceutical composition and an expression vector.
Group IV, claim(s) 30, a method to improve solubility and yields in production of recombinant proteins.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: A special technical feature does not link the claims as the prior art reveals compounds which prevent protein aggregation (Wisniewski et al (1993) Biochem. Biophys. Res. Comm., 192, 359-365. There is no unity of invention absent a special technical feature which links the claims as provided in PCT Rule 13.2.